Cellular Biology

Bone Sialoprotein Mediates Human Endothelial Cell Attachment and Migration and Promotes Angiogenesis

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Abstract—Bone sialoprotein (BSP) is a secreted glycoprotein primarily found in sites of biomineralization. Recently, we demonstrated that BSP is strongly upregulated in osteotropic cancers and particularly those that exhibit microcalcifications. BSP contains an Arg-Gly-Asp (RGD) motif found in other adhesive molecules that interact with cellular integrins. In bone, BSP has been shown to mediate the attachment of osteoblasts and osteoclasts via $\alpha_v\beta_3$ integrin receptors. Ligands for $\alpha_v\beta_3$ integrin are considered to play a central role during angiogenesis. Therefore, we used human umbilical vein endothelial cells (HUVECs) to study the potential role of BSP in angiogenesis. We found that purified eukaryotic recombinant human BSP (rhBSP) is able to promote both adhesion and chemotactic migration of HUVECs in a dose-dependent manner. These interactions involve HUVEC $\alpha_v\beta_3$ integrin receptors and the RGD domain of BSP. Indeed, HUVECs attach to a recombinant BSP fragment containing the RGD domain, whereas this response is not observed with the same fragment in which RGD has been mutated to Lys-Ala-Glu (KAE). A cyclic RGD BSP peptide inhibits both adhesion and migration of HUVECs to rhBSP. Moreover, anti-$\alpha_v\beta_3$ but not anti-$\alpha_v\beta_5$ monoclonal antibodies also prevent BSP-mediated adhesion and migration of HUVECs. We observed that both rhBSP and the RGD BSP recombinant fragment stimulated ongoing angiogenesis on the chorioallantoic chick membrane assay. BSP angiogenic activity was inhibited by anti-$\alpha_v\beta_3$ antibody, and the KAE BSP fragment was inactive. Our findings represent the first report implicating BSP in angiogenesis. BSP could play a critical role in angiogenesis associated with bone formation and with tumor growth and metastatic dissemination. (*Circ Res*. 2000;86:885-891.)

Key Words: bone sialoprotein ■ angiogenesis ■ integrins

Angiogenesis, the formation of new blood capillaries from preexisting vessels, is one of the most critical processes that occur in vertebrates. It is important for growth and tissue repair and is also implicated in several pathologies, including psoriasis, arthritis, and cancer.[1] On stimulation by an angiogenic factor, endothelial cells first proliferate, then degrade the subendothelial basement membrane, and migrate toward the underlying extracellular matrix (ECM) (for review, see Reference 2). Angiogenesis depends not only on angiogenic factors but also on vascular adhesion molecules. A number of studies have demonstrated the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors in endothelial cell biology and angiogenesis.[2] These integrins are receptors for multiple ECM ligands that contain the Arg-Gly-Asp (RGD) cell-binding sequence.[3,4] Interestingly, $\alpha_v\beta_3$ receptors are not expressed on quiescent blood vessels but are highly upregulated during angiogenesis.[5] Consequently, upregulation of $\alpha_v\beta_3$ in angiogenic endothelial cells suggests that these cells could interact with new ECM ligands not associated with quiescent endothelial cells. Interactions of endothelial cells via $\alpha_v\beta_3$ receptors are thought to be essential for the progres-

sion of angiogenesis. Interfering with them by either specific anti-integrin antibodies or RGD-containing peptides induces endothelial cell apoptosis and aborts the capillary formation process.[5–8]

Bone sialoprotein (BSP) is an acidic glycoprotein synthesized by osteoblasts and osteoclasts and other skeleton-associated cell types.[9,10] We have recently demonstrated that BSP is strongly upregulated in carcinomas that exhibit microcalcifications and that metastasize to bone with high frequency.[11–15] One characteristic feature of the protein is the presence of an RGD sequence that is situated near the carboxy-terminus[16] and is recognized by the $\alpha_v\beta_3$ integrin receptor.[17] BSP has been shown to mediate the attachment of fibroblasts,[18] osteoblastic cells,[17] and osteoclasts[19] to solid surfaces. Moreover, it has recently been demonstrated that BSP promotes human breast cancer cell adhesion, proliferation, and migration through integrin-mediated interactions.[20]

Because $\alpha_v\beta_3$ integrin ligands are believed to play key roles during angiogenesis, we explored the possibility that BSP could be involved in the process leading to the formation of new blood vessels. We investigated the potential contribution

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of BSP to human endothelial cell adhesion and migration in vitro. In addition, we evaluated the effect of BSP on in vivo angiogenesis by using the chicken chorioallantoic membrane (CAM) assay.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described.[21] The endothelial nature of the cells was confirmed by staining with an antiserum to von Willebrand factor (Dako).

rhBSP and Synthetic Peptides

Full-length human BSP (clone B6-5g)[22] was subcloned into plasmid pACCMV.pLpA,[23] whose promoter had been replaced with E1A. A successful replication-deficient viral recombinant that caused over-expression of human BSP was used to infect human marrow stromal cells (1×10^6 cells per dish, passage 3, in 10-cm dishes were infected with 10 000 plaque-forming units per cell). BSP from the serum-free media was purified to >95% by standard high-performance liquid anion-exchange chromatography. Protein sequencing verified the amino-terminus, and Western blot analysis with LF-83 antiserum[24] showed that the carboxy-terminus was likely fully intact. Recombinant human BSP (rhBSP) carboxy-terminal domains (258E to 317Q) either with the natural RGD (rRGD) or with Lys-Ala-Glu (KAE) substituted (rKAE) were made and purified from bacteria as previously described.[25] RGD and Arg-Gly-Glu (RGE) cyclic BSP peptides, called cRGD and cRGE, respectively, were synthesized by using an automated solid-phase peptide synthesizer as described earlier.[26] The structure of these peptides is represented in the Table.

Cell Attachment Assay

Bacteriological 96-well plates (Greiner) were coated with rhBSP or vitronectin as described previously.[24,27] HUVECs were incubated at 37°C for 2 hours in the precoated wells. Attached cells were stained with crystal violet, and the incorporated dye was measured by reading absorbance at 560 nm. For blocking experiments, either cRGD and cRGE cyclic peptides (1 to 100 nmol/L) or anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ antibodies (Chemicon) and mouse purified IgG (10 μg/mL, Serotec) were added to cell suspensions.

Cell Migration Assay

rhBSP diluted in RPMI 1640–0.1% BSA to final concentrations ranging from 50 to 1000 nmol/L was used as a chemoattractant in the bottom of a modified Boyden chemotaxis chamber (Neuroprobe Inc). For testing the effects of a concentration gradient, rhBSP was also placed in both the top and bottom chambers or in the top chamber with the cells. RPMI 1640–0.1% BSA was used as negative control. HUVECs that had traversed the filter after an overnight incubation at 37°C were stained and counted. In blocking experiments, either cRGD or cRGE (2.5 μmol/L), $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-blocking antibodies, or mouse purified IgG1 (20 μg/mL) was added to the cell suspensions.

Structure and Abbreviation of BSP Recombinant Fragments and Synthetic Cyclic Peptides Used in Study

BSP Peptide Designation	Abbreviation	Structure
RGD recombinant fragment	rRGD	258E———EP**RGD**NYR———317Q
KAE recombinant fragment	rKAE	258E———EP**KAE**NYR———317Q
RGD synthetic cyclic peptide	cRGD	DPA-EP**RGD**NYRCysNH₂ └———CM———┘
RGE synthetic cyclic peptide	cRGE	C-EP**RGE**NYR-BPA └——CM——┘

Single-letter codes are used for amino acids except for cysteinamide (CysNH₂). DPA indicates α,β-L-diaminopropionic acid; BPA, N^β-bromoacetyldiaminopropionic acid; and CM, S-carboxymethylcysteine.
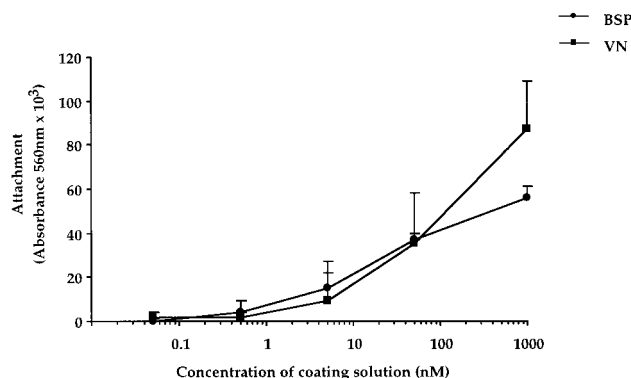

Figure 1. Dose-dependent attachment of human endothelial cells to BSP. Adhesion assays were performed with increasing concentrations of rhBSP coated onto plastic. VN was used as a positive control. Cells were allowed to adhere for 2 hours at 37°C and were quantified as described in Materials and Methods. Cell attachment to PBS-treated wells was subtracted as background. Data represent mean±SD of 6 replicates of a representative experiment. The experiment was repeated 3 times.

Chicken CAM Assay

Fertilized Lohman-selected White Leghorn eggs were incubated at 37°C. On day 8, Silastic rings were placed on the chick embryo CAM surface. rhBSP (15 μmol/L) and rRGD or rKAE fragments (20 and 100 μmol/L) were applied inside the rings. Vehicle alone (PBS) and basic fibroblast growth factor (bFGF, 0.5 μmol/L) were used as negative and positive controls, respectively. In other experiments, the anti-$\alpha_v\beta_3$ antibody LM609 (15 μg) was added to the ring to evaluate its effect on vascular development in the presence of BSP. CAMs were examined and photographed in ovo with a Leica DMLM microscope (Van Hopplynus). A vascular index was determined by counting all discernible vessels traversing the ring as described[28] and was expressed as the relative increase of the number of vessels in the different experimental conditions compared with the control PBS ring.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

BSP Mediates the Adhesion of HUVECs in an RGD-Dependent Manner

To investigate the possibility that BSP could serve as an adhesion substrate for endothelial cells, we produced and purified an rhBSP by using an adenovirus-BSP cDNA construct transfected into human marrow stromal cells. HUVECs were tested for their ability to adhere to various concentrations of rhBSP or vitronectin (VN), used as a positive control.[29] HUVECs attached to rhBSP in a dose-dependent manner. Attachment to BSP was comparable to that observed with VN substrate (Figure 1). BSP contains an RGD motif, which has been identified as one common integrin recognition site mediating cell-ECM interactions.[30] To evaluate the potential role of the RGD domain in the attachment of HUVECs to BSP, we performed attachment assays by using 2 rhBSP fragments made in *Escherichia coli* encompassing the RGD motif (amino acids 258E to 317Q). The first recombinant fragment contains an intact RGD sequence (rRGD); in the second fragment, this motif has been changed to KAE (rKAE). HUVECs adhered and spread on rRGD, whereas they did not show any attachment to rKAE, indicat-

ing that the adhesion of HUVECs to BSP is most likely mediated through interactions with the RGD motif (data not shown). To further investigate this hypothesis, we examined whether exogenously added BSP synthetic peptides could modulate the adhesion of HUVECs to rhBSP. RGD-containing cyclic peptides are generally more potent than are the corresponding linear peptides, suggesting that their tertiary structure is important for their ability to interfere with cellular adhesion in vitro.[31,32] Therefore, we used a cyclic peptide containing the RGD consensus sequence flanked by BSP amino acids (cRGD), which has been recently shown to inhibit MDA-MB-231 cell adhesion to bone sections.[27] When cRGD was added to HUVECs at concentrations ranging from 1 to 100 nmol/L, a dose-dependent inhibition of cell attachment to rhBSP was observed, with a nearly complete inhibition (93%) at 100 nmol/L (Figure 2A). The use of a control cyclic peptide in which the RGD sequence is substituted by RGE (cRGE) did not induce any inhibition of the attachment (Figure 2A). These data demonstrate that the RGD domain of BSP plays an important role in HUVEC adhesion. To further define which cell surface integrins might be responsible for this effect, blocking studies were performed with the use of well-characterized monoclonal antibodies. Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been described as potential receptors for BSP. Because angiogenic endothelial cells express these 2 integrins (fluorescence-activated cell sorter analysis, data not shown), we tested the effect of monoclonal antibodies directed against $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the adhesion of HUVECs to BSP. Incubation of HUVECs with LM609 anti-$\alpha_v\beta_3$ integrin antibody inhibited their adhesion to immobilized rhBSP (Figure 2B). No significant effect was observed when HUVECs were incubated with P1F6 anti-$\alpha_v\beta_5$ monoclonal antibody or with normal mouse IgG used as a control (Figure 2B). These data suggest that BSP mediates HUVEC attachment primarily through molecular interaction with the $\alpha_v\beta_3$ receptor.

BSP Stimulates HUVEC Migration In Vitro Through Its RGD Sequence

The ability of BSP to mediate HUVEC migration was evaluated by using a modified Boyden chamber assay. When placed in the lower chamber, rhBSP stimulated HUVEC migration in a dose-dependent manner (Figure 3). There was no migration to BSA in the lower chamber (data not shown). To determine the importance of a concentration gradient for the migratory effects of BSP, cell migration was also evaluated when the protein was placed either in the top chamber only or in both chambers. BSP has chemotactic properties, inasmuch as placing this molecule in both chambers at the same concentration reduced maximal migration by 43% (Figure 4A). However, because the absence of a concentration gradient in this experiment did not totally abolished cell migration, we can also conclude that BSP exhibits chemokinetic ability toward endothelial cells.

We next used cRGD and cRGE peptides to investigate whether the migration of HUVECs toward BSP depends on the presence of the RGD motif. Incubation of endothelial cells with cRGD resulted in a dramatic inhibition of cell migration (Figure 4B). No effect was observed when HUVECs were treated with the control cRGE peptide. To
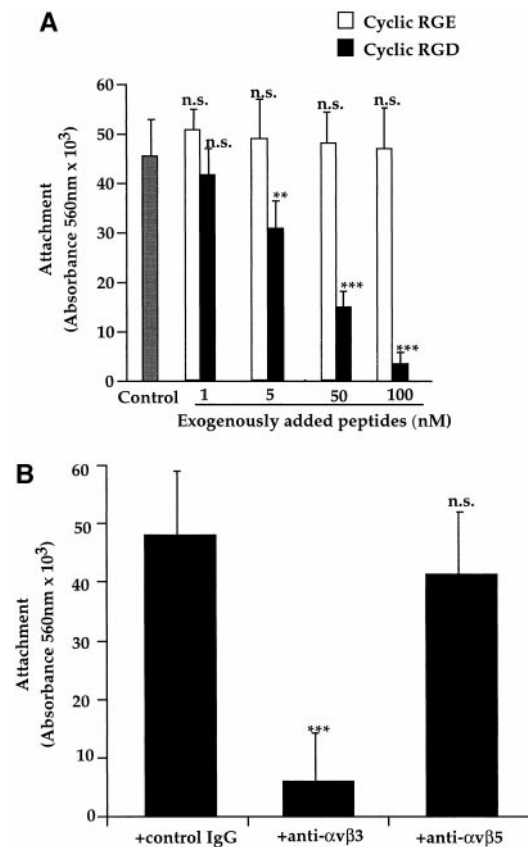

Figure 2. Inhibitory effects of exogenously added cyclic BSP RGD peptide and anti-$\alpha_v\beta_3$ antibodies on human endothelial cell adhesion to BSP. A, HUVECs were tested for adhesion to bound rhBSP (50 nmol/L) in the presence of various concentrations of cRGD or cRGE synthetic peptides. cRGD peptide inhibited in a dose-dependent manner the adhesion of HUVECs to BSP, which confirms the RGD-dependent nature of HUVEC attachment. In contrast, cRGE peptide used at the same concentrations did not change adhesion of these cells to BSP. The control (stippled bar) represents the attachment to rhBSP in the absence of exogenously added peptides. B, Cells were allowed to adhere to rhBSP (50 nmol/L) in the presence of anti-$\alpha_v\beta_3$ (LM609) or anti-$\alpha_v\beta_5$ (P1F6) antibodies or normal mouse IgG used as a control. A functional $\alpha_v\beta_3$ integrin is necessary for the adhesion of HUVECs to BSP, whereas $\alpha_v\beta_5$ did not significantly intervene in this attachment. Attached cells were stained and quantified as described in Materials and Methods. Cell attachment to PBS-treated wells was subtracted as background. The mean±SD of 6 replicates of a representative experiment is shown. Both blocking experiments were repeated 3 times. **$P=0.005$ and ***$P=0.0001$ vs control by 2-sided t test. n.s. indicates not significant.

determine whether $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ plays a role in the endothelial cell migration response toward BSP, we used the corresponding integrin-blocking antibodies in migration assays. Incubation of HUVECs with LM609 anti-$\alpha_v\beta_3$ antibody inhibited cell migration by 84% (Figure 4C), demonstrating that endothelial cells use this integrin to interact with BSP. Neither anti-$\alpha_v\beta_5$ monoclonal antibody nor a normal mouse IgG control used at the same concentration modulated cell migration (Figure 4C).

BSP Is Angiogenic in the Chick CAM Assay

The observation that BSP mediates adhesion and migration responses in HUVECs urged us to investigate the possibility
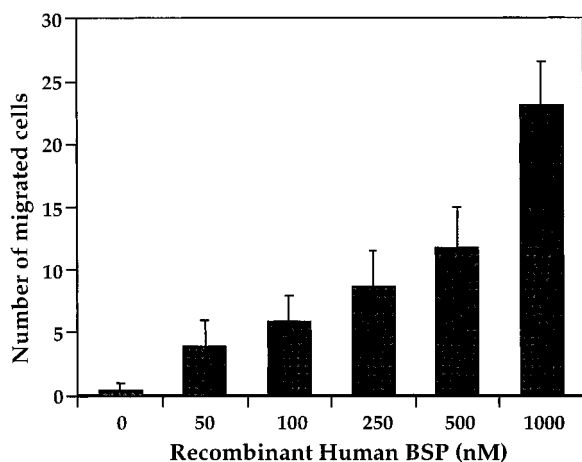
Figure 3. Human endothelial cells migrate in a dose-dependent manner to BSP. HUVEC migration on increasing concentrations of rhBSP was determined by use of a modified Boyden chamber. Cells were allowed to traverse the membrane for 12 hours, and the cells were then stained and counted as described in Materials and Methods. Each bar represents mean±SD of the total number of migrated cells within 4 replicate wells. The experiment was repeated 3 times.

that it could promote angiogenesis. Therefore, we have evaluated the effect of rhBSP as well as rRGD on the ongoing angiogenesis process in the in ovo CAM assay. After 2 days of incubation, both rhBSP and rRGD elicited an angiogenic response visible with the microscope as a brushlike formation of blood vessels. The effect was clearly observed with 15 μmol/L rhBSP in the bFGF positive control ring, whereas around the control ring containing vehicle alone (PBS), no vascular growth was observed (Figure 5A). The role of integrin activation in BSP-mediated angiogenesis was evaluated in 2 ways. First, anti-$\alpha_v\beta_3$ monoclonal antibody with blocking activity was applied in the CAM assay along with rhBSP, and it was able to abrogate the angiogenic response observed with BSP alone (Figure 5A). The quantification of these effects is presented in Figure 5B. In a separate series of experiments, the rRGD BSP fragment was compared with the corresponding mutated rKAE fragment. Whereas the rRGD fragment induced a weak angiogenic activity at 20 μmol/L and a stronger effect at a higher concentration (100 μmol/L), the rKAE fragment had no visible effect at either concentration (data not shown). These data suggest that the angiogenic effect of BSP occurs via endothelial cell integrin activation.

Discussion

BSP is a sialic acid–rich adhesive ECM protein containing an RGD cell-binding sequence. BSP was first considered to be highly specific to mineralized tissue, in which it is involved in the early process of mineralization and in bone resorption.[33] The observation that BSP is also expressed in trophoblastic tissue[10] and several human cancers[12,14,15,34] suggests that this glycoprotein may have additional biological functions. In the present study, we demonstrate for the first time that BSP plays a role in angiogenesis. The angiogenic process depends on specific molecular interactions between endothelial cells and components of the ECM in which integrins play a key role.[2] Among the wide spectrum of integrin subunit combi-
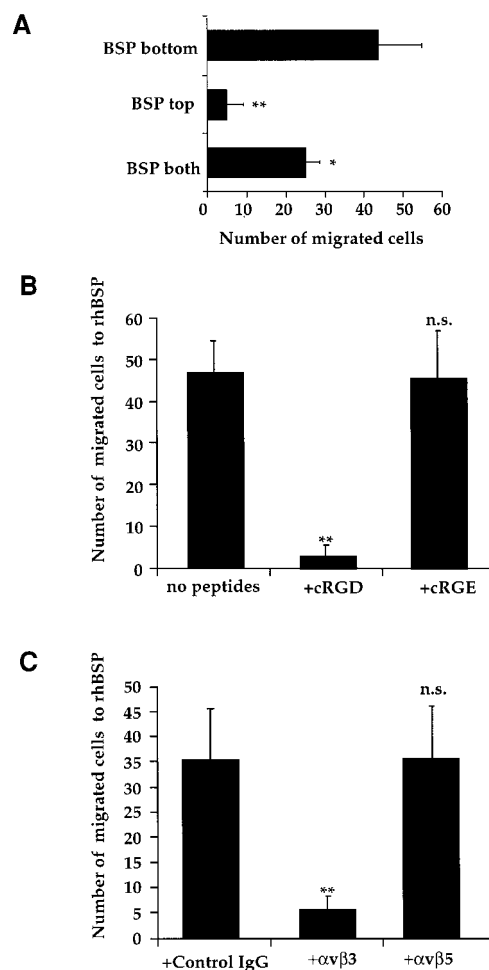

Figure 4. BSP mediates endothelial cell migration through $\alpha_v\beta_3$ integrin receptor. Modified Boyden chamber chemotaxis assays were performed with rhBSP at 250 nmol/L. A, rhBSP was placed in the bottom chamber (BSP bottom), in the top chamber with the cells (BSP top), or in both the top and bottom chambers (BSP both). B, Migration of HUVECs to rhBSP was evaluated in the presence of cRGD or cRGE BSP synthetic peptides. Control (no peptides) represents the number of migrated cells to rhBSP in the absence of exogenously added peptides. C, Cells were allowed to migrate to rhBSP in the bottom chamber in the presence of anti-$\alpha_v\beta_3$ (LM609), anti-$\alpha_v\beta_5$ (P1F6), or normal mouse IgG. Each bar represents mean±SD of total number of migrated cells within 4 replicate wells. *P=0.05 and **P=0.005 vs control by 2-sided t test. n.s. indicates not significant.

nations, abundant data have identified $\alpha_v\beta_3$ as one of the major ECM receptors involved during angiogenesis. Endothelial cells engaged into the process of new capillary formation express $\alpha_v\beta_3$ receptors at their surface.[5] The ligation of this integrin to an appropriate ligand induces a survival-signaling pathway critical for the completion of angiogenesis.[6] Because BSP is a known ligand for $\alpha_v\beta_3$ integrin receptors expressed at the surface of osteoclasts[19] and osteoblasts,[17] it is not surprising that this protein represents an appropriate substrate for endothelial cells. Indeed, we demonstrate in the present study that adhesion and migration of HUVECs to BSP are dependent on the interaction of endothelial cell surface $\alpha_v\beta_3$ receptors with the RGD motif of BSP. The observation that the RGD recombinant fragment of BSP
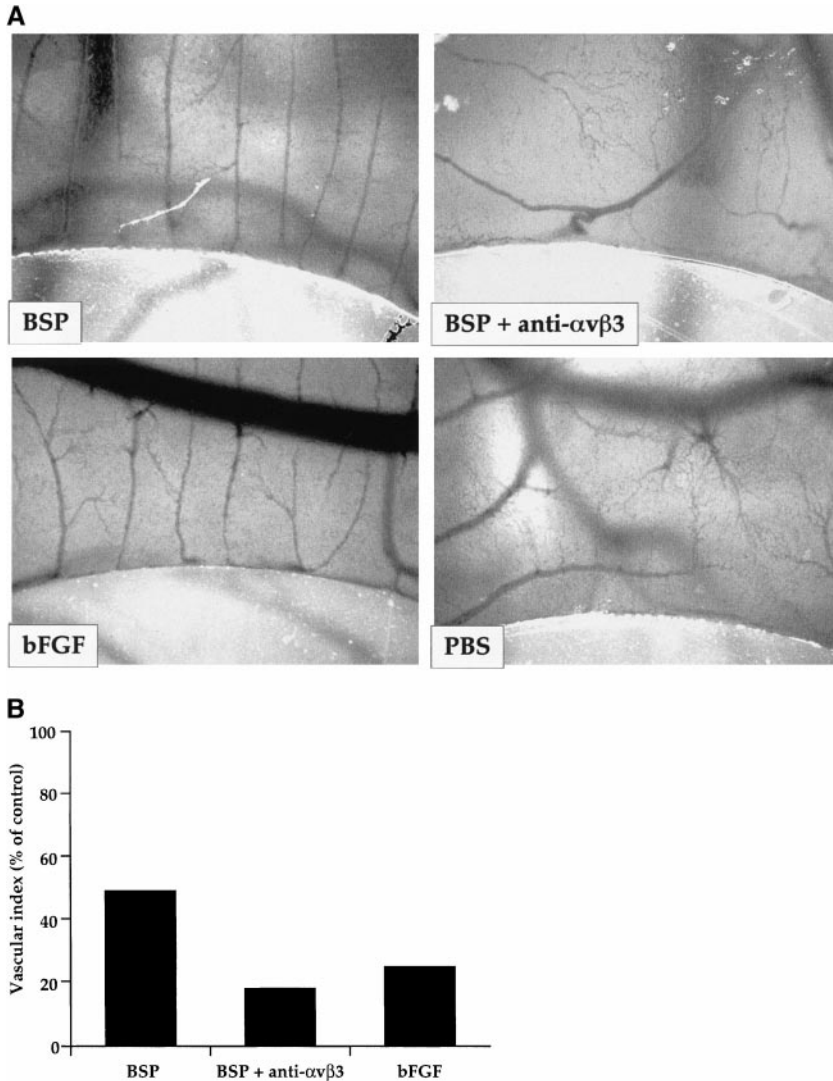
Figure 5. BSP is angiogenic in the chick CAM assay. A, rhBSP (BSP) at 15 μmol/L, bFGF at 0.5 μmol/L, or PBS was applied to the chick CAM, and its effect on vascular development was evaluated. In some experiments, LM609 $\alpha_v\beta_3$-blocking antibody (15 μg) was added simultaneously with BSP in the ring to examine its ability to modify the BSP angiogenic response. A radially arranged "spoke-wheel" pattern is present around BSP and bFGF rings, whereas no vascular response is observed in control CAM with PBS and in the presence of $\alpha_v\beta_3$-blocking antibody. CAMs were photographed after 48 hours of incubation. Experiments were repeated at least twice, with a minimum of 8 embryos per condition. Original magnification $\times$60. B, Quantification of a representative CAM assay indicates that BSP is angiogenic via an $\alpha_v\beta_3$-dependent mechanism. Relative vascular index was determined as described in Materials and Methods.

obtained from bacteria sustained the adhesion and migration of endothelial cells as efficiently as did the intact human recombinant molecule indicates that this biological activity is primarily associated with the RGD motif and does not significantly depend on posttranslational modifications of BSP (eg, sialylation and phosphorylation). Cell adhesion and migration responses to BSP are comparable to those observed at similar concentration ranges with a classical integrin RGD ligand, eg, VN. Experiments in the present study show that both responses are primarily mediated through BSP binding to $\alpha_v\beta_3$ and not to $\alpha_v\beta_5$.

BSP shares structural similarities at both genomic and protein levels with another noncollagenous bone matrix called osteopontin.[35] Interestingly, osteopontin was also found to stimulate endothelial cell adhesion and migration through $\alpha_v\beta_3$ receptors.[36,37] Integrin $\alpha_v\beta_3$ is capable of recognizing a number of ECM proteins that contain an RGD adhesive motif. However, in the context of tumor-associated angiogenesis, BSP may represent a unique appropriate ligand for $\alpha_v\beta_3$ that is highly expressed by cancer cells at both the primary and the metastatic sites. This hypothesis is supported by the fact that BSP is strongly upregulated in many cancer cell types and that its expression in primary breast and prostate cancer tumors is associated with progression of the disease.[15,38]

One of the most exciting observations in the present study is that in addition to being a functional ligand for $\alpha_v\beta_3$, BSP appears to be able to stimulate angiogenesis. Indeed, in the CAM assay, the angiogenic response to BSP was comparable to that obtained with bFGF, a potent angiogenesis inducer. The finding that BSP stimulates angiogenesis was unexpected because there is no evidence that integrin ligands can initiate the angiogenic process in a fashion similar to angiogenic growth factors. Most $\alpha_v\beta_3$ ligands known to stimulate the adhesion and migration of endothelial cells, such as fibronectin and VN, are not angiogenic in the CAM assay. However, it has been recently reported that Del1, a novel RGD-containing ECM protein, initiates angiogenesis through a molecular pathway(s) that has not yet been elucidated.[39] Our finding that the anti-$\alpha_v\beta_3$ monoclonal antibody inhibited BSP-induced angiogenesis in the CAM assay indicates that $\alpha_v\beta_3$ activation is, at least in part, implicated in this process. Numerous studies have concentrated on identifying molecules involved in the initiation phase of tumor angiogenesis.

These studies led to the conclusion that tumor cells can release angiogenic molecules, such as bFGF and vascular endothelial growth factor. We believe that BSP may be released by cancer cells to initiate the formation of new blood vessels, an event essential for tumor progression.

The new information gained in the present study about the angiogenic activity of BSP could also be useful when applied to understanding the potential function(s) of BSP in bone physiology. BSP was originally identified in bone, where it represents 15% of the noncollagenous proteins found in the mineralized matrix.[9] Most bones of the skeleton are first formed as avascular cartilage rudiments during osteogenesis. Chondrocytes, cells that constitute cartilage, undergo a program of hypertrophy, calcification, and cell death. When vascularization occurs in the hypertrophic cartilage, osteoblasts and osteoclasts are recruited, and the cartilage is progressively converted into bone (for review, see Reference 40). It is believed that hypertrophic chondrocytes and osteoblasts express angiogenic molecules that are responsible for the vascular invasion accompanying the calcification of bone matrix. Indeed, a number of angiogenic factors have been reported in cartilage.[41–43] Interestingly, BSP is known to be specifically associated with the early phases of bone formation, and the detection by electron microscopy of this protein corresponds to the sites of early mineral deposition.[44] Moreover, BSP protein and its mRNA are upregulated in hypertrophic chondrocytes and mature osteoblasts.[9,10] Altogether, these observations and the present results suggest that BSP could support the vascular invasion process, which is fundamental to bone formation.

Although our findings provide novel insight into elucidating BSP functions, further studies are warranted to establish the relevance of BSP angiogenic properties in bone formation and tumor growth and metastasis.

Acknowledgments

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